Description of the first Schmallenberg disease outbreak in Spain and subsequent virus spreading in domestic ruminants

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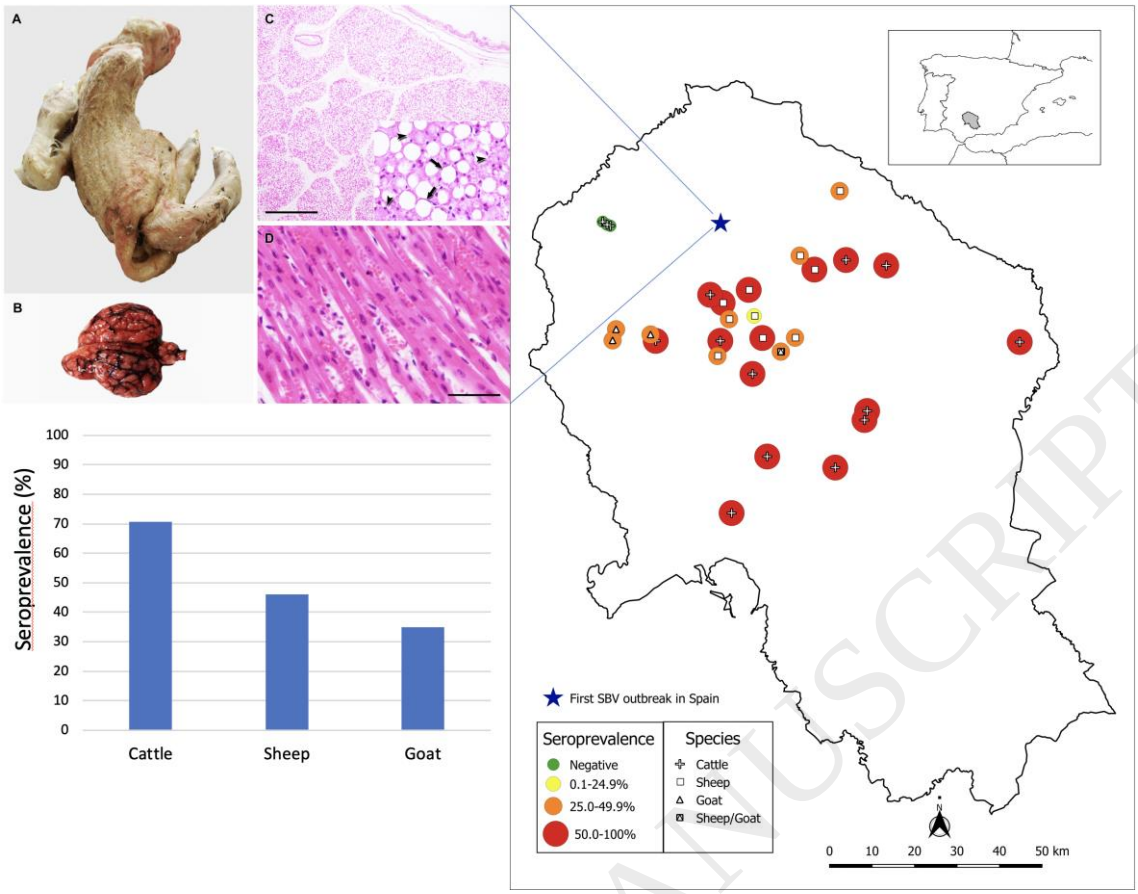
Graphical abstract



Highlights

- First Schmallenberg disease outbreak in Spain
- Widespread circulation of SBV in domestic ruminants one year after the first outbreak
- Species (cattle), age (adult) and absence of animal insecticide treatment were risk factors associated with SBV infection
- Surveillance in extensively managed domestic ruminants should be implemented in Spain

Abstract

Schmallenberg disease (SBD) is an emerging disease transmitted mainly among ruminant species by biting midges of the genus *Culicoides*. Since the Schmallenberg virus (SBV) was first identified in Germany in late 2011, it rapidly spread to other European countries. The aims of the present study were to describe the first SBD outbreak in Spain and to assess the spread and risk factors associated with SBV infection in domestic ruminants from nearby farms during the following year. In March 2012, one malformed stillborn lamb from a sheep farm located in Cordoba province (Southern Spain) was subjected to necropsy. Pathological compatible lesions and molecular analyses confirmed the first SBV infection in Spain. Afterwards, serum samples from 505 extensively reared domestic ruminants from 29 farms were analysed using both blocking ELISA and virus neutralization test against SBV. The overall seroprevalence was 54.4% ($CI_{95\%}$: 50.0-58.7). Antibodies were detected in 70.6%, 46.0% and 34.8% of cattle, sheep and goats, respectively. A generalized estimating equation model indicated that the main risk factors associated with SBV infection were: species (cattle), age (adult), and absence of animal insecticide treatment. Pathological and molecular results confirmed the presence of SBV in Spain few months after it was firstly identified in Germany. The seroprevalence detected indicates a widespread circulation of SBV in nearby domestic ruminant farms one year after this first outbreak was reported in Spain. Further studies are warranted to determine the spatio-temporal trend of SBV in domestic ruminants in this country.

Keywords: First outbreak; Schmallenberg virus; Serosurvey; Risk factors; Spain

1. Introduction

Schmallenberg virus (SBV) is an emerging arthropod-borne virus belonging to the Simbu serogroup (genus *Orthobunyavirus*; family *Peribunyaviridae*) [1], which is transmitted mainly among ruminant species by biting midges of the genus *Culicoides* [2]. Schmallenberg disease (SBD) is characterized by non-specific clinical signs including fever, milk yield reduction, diarrhoea as well as reproductive disorders. In pregnant females, SBV infection can lead to abortions, stillbirths and congenital malformations in newborns [2].

Since the virus was first identified in cattle in Germany in September 2011 [3], it has been detected in domestic and wild ruminants in most European countries. Currently, SBV is considered endemic within Europe [reviewed in 4,5] and sporadic outbreaks and viral circulation have been reported in other continents, including Asia and Africa [6-8]. During the last few years, significant productive losses, international trade restrictions and veterinary costs have been associated to SBD in the affected countries [9].

The spatio-temporal trend of SBV has been monitored in different European countries [4,10-13]. In Spain, the first SBV outbreak was reported by the Ministry of Agriculture, Food and Environment Affairs on March 2012 [14], but pathological analyses were not conducted in the affected animal. Moreover, although SBV circulation was reported in domestic ruminants during the following years [15,16], the epidemiological information about the virus is still very limited in this country. Hence, in this study we describe the first outbreak of SBD in Spain and assess the spread and risk factors associated with SBV infection in domestic ruminants from farms in close proximity to the first affected flock.

2. Materials and methods

On March 7th, 2012, one malformed stillborn lamb from an extensively managed mixed small ruminant flock (644 Merino sheep and 12 crossbred goats), located in the province of Cordoba (Southern Spain) (38º25'35.834'' N, 5º4'46.202'' W), was remitted to the Animal Health Laboratory of the University of Cordoba (Figure 1A). A systematic necropsy was performed, and the organs were macroscopically examined to assess the presence of gross lesions. A panel of tissue samples was collected for both histopathological and molecular studies. Samples of skeletal muscles, lung, heart, liver and kidney were fixed in 10% neutral buffered formalin for 24 h at 22±2ºC, and then, dehydrated in a graded series of ethanol, immersed in xylol, and embedded in paraffin wax using an automatic processor. Sections were cut at 4 µm and stained with haematoxylin and eosin (HE), following standard procedures. Blood, amniotic fluid and fresh tissue samples (placenta, brain, lung, heart, liver, kidney and small intestine) were collected for molecular analysis and stored at -80ºC until tested. Viral RNA was extracted using a commercial kit (MagAttract® 96cador® Pathogen Kit QIAGEN, Hilden, Germany) and tested in duplicate using a real time RT-PCR in order to detect a conserved region within the S segment of the SBV genome as described [17].

During 2013, in order to assess the spread of SBV, a serosurvey study was carried out in domestic ruminants in the same region where the first Spanish SBV outbreak was confirmed (Figure 2). The sample size was based on an estimated prevalence of 50.0%, a 95% confidence interval ($CI_{95\%}$), an accepted error of 5% and the large population of domestic ruminants in the study region (n >10,000). Farms were randomly selected within a radius up to 75.0 km from where the malformed stillborn lamb was found (Figure 2). The number of ruminants sampled within each farm (20 whenever possible) was chosen to ensure a 95% probability of detecting at least one positive animal for an assumed minimum within-herd prevalence of 15%. Finally, a

total of 505 extensively reared domestic ruminants including 213 cattle, 203 sheep and 89 goats from 29 farms (15 cattle herds, 10 sheep flocks, three goat flocks and one mixed small ruminants farm) were included in the study. The size of the sampled farms ranged from 10 to 750 animals (median 80; mean 186).

Blood samples were obtained by both puncture of the jugular (sheep and goats) and medial caudal (cattle) veins. Samples were centrifuged for 15 min at 1800 *g* for serum separation and stored at -20ºC until serological analyses. The presence of antibodies against the N protein of SBV was analysed using a commercial blocking ELISA (bELISA; INgezim Schmallenberg Compac®, INGENASA, Madrid, Spain). Sensitivity and specificity values provided by the manufacturers are 98% and 99%, respectively. Positive and doubtful bELISA sera were tested by virus neutralisation test (VNT) using the BH80/11–4 isolate (provided by the Friedrich-Loeffler-Institut, Isle of Riems, Germany) as previously described [18]. Titres were expressed as the reciprocal of the highest dilution that neutralised 100 tissue culture infective doses (100 $TCID_{50}$) in Vero cells. Sera that showed neutralisation (absence of cytopathic effect) at dilutions ≥1:5 were considered positive. Seroprevalence to SBV was determined from samples positive by both bELISA and VNT.

Epidemiological information related to the sampled animals, management, production parameters, environmental data and biosecurity measures was gathered by direct interview with farmers. Differences between seropositivity to SBV and explanatory variables were analysed using Chi-square and Fisher's exact test to assess the relevance of the explanatory variables in the risk of an animal being exposed to SBV. All statistically significant variables (likelihood ratio and Wald test, $P<0.10$) in the bivariate analysis were selected as potential risk factors. Cramer's V coefficient between pairs of variables was computed to prevent collinearity. Finally, a generalized

estimating equation (GEE) analysis was performed to study the effect of the variables previously selected on the basis of bivariate analysis. The number of seropositive animals was assumed to follow a binomial distribution and the "farm" was included as random effect. The model was re-run until all remaining variables presented statistically significant values (likelihood-ratio Wald's test, $P<0.05$) and a potential relationship with the response variable existed. The fit of the models was assessed using a goodness-of-fit test [19]. Statistical analyses were performed using SPSS v. 25.0 software (IBM Corp., Armonk, NY, USA).

3. Results

Even though only one foetus was submitted to the laboratory, the farmer of the affected flock revealed that more abortions and newborn lambs with similar congenital malformations were found in this flock at the same time. Considering the size of the foetus (48 centimetres long), the abortion was estimated between 135 and 150 days of gestation. Macroscopically, the foetus displayed different musculoskeletal defects such as skeletal muscular atrophy, brachygnathia inferior, general ankylosis, arthrogryposis multiplex congenita and curvature of the spine (torticollis and kyphoscoliosis) (Figure 1A). Moreover, a severe congestion of the cerebral meninges and cerebellar hypoplasia were observed in gross examination (Figure 1B). Histopathologically, skeletal muscles showed a severe muscle hypoplasia with replacement of muscle tissue by adipose tissue (Figure 1C). Vascular alterations were observed in heart and kidney, characterized by oedema in the cardiac muscle with intense congestion of interfibrillar capillaries and renal medulla (Figure 1D). Lung presented foetal atelectasis with severe aspiration of meconium. The remaining fixed organs lacked significant histopathological lesions. SBV-RNA was detected in placenta (threshold cycle; C_t=27), blood (C_t=29), heart (C_t=30), amniotic fluid (C_t=31) and lung (C_t=34). SBV-RNA was also confirmed in

brain samples submitted to the National Central Laboratory of Veterinary Medicine (CLVM) in Algete (Madrid, Spain).

A total of 281 out of 505 analysed ruminants were positive by bELISA. Five of them were negative by VNT and three could not be analysed due to cytotoxicity. Hence, the overall individual seroprevalence was 54.4% (273/502; $CI_{95\%}$: 50.0-58.7). By species, 70.6% (149/211) of cattle, 46.0% (93/202) of sheep and 34.8% (31/89) of goats had specific antibodies against SBV. Moreover, seropositivity was detected in 89.7% (26/29; $CI_{95\%}$: 78.6-100.0) of the analysed farms (Figure 2). The distribution of SBV seropositivity according to the explanatory variables is shown in Table 1. The GEE model showed that the main risk factors associated with SBV infection in domestic ruminants were: species (cattle), age (adult) and absence of animal insecticide treatment (Table 2).

4. Discussion

The main macroscopic findings observed in the present study (arthrogryposis, curvature of the spine, brachygnathia inferior, cerebral congestion and cerebellar hypoplasia) are in agreement with those reported in SBV infections in small ruminants in other European countries [20,21]. Other teratogenic effects including different cavitational lesions of the central nervous system (porencephaly, hydranencephaly, hydrocephalus, micrencephaly, macrocephaly), reduced size of the spinal cord, domed or flattened skull, cardiac interventricular septal defect, unilateral hydronephrosis and colonic atresia, have also been associated with SBV infections in these species [22,21], but were not observed in our study.

Severe skeletal muscle hypoplasia with replacement of myocytes by adipose tissue detected in our study has been previously proposed as the only significant lesion

detected in peripheral organs of both SBV-infected sheep and cattle stillborns and newborns [16,21-23]. The presence of foetal atelectasis with mild aspiration of meconium as well as the vascular alterations in other peripheral organs have not only been associated with SBV [23], but also with abortions produced by other aetiological agents causing perinatal mortality syndrome in ruminants [24]. The lack of other significant lesions in the remaining peripheral organs is in line with previous reports [21-23]. The main microscopic lesions caused by SBV infection at central nervous system level include cerebral cavitation, depletion of the cerebellar granular cell layer and loss of ventral motor neurons in the spinal cord [22,23]. Unfortunately, because the brain was submitted to the CLVM, the histopathological analysis of this organ could not be carried out.

SBV-RNA was detected in placenta, blood, heart, amniotic fluid, lung and brain, which is consistent with previous reports on both natural and experimental SBV infections [17,25,26]. Compatible pathological lesions together with the molecular results confirm the first SBV outbreak in Spain on March 2012 [14]. The absence of external replacement in the affected flock as well as in 24 of the 26 (92.3%) seropositive farms suggest local circulation of the virus in the study area between 2012 and 2013. Given the observed lesions are consistent with those induced by SBV during the second month of pregnancy [27], and because the age of the foetus was between 135 and 150 days, the infection of the pregnant sheep may have occurred between mid-November and mid-December 2011. Although the movement of infected animals from Central Europe to farms geographically close to the affected flock cannot be ruled out, our results support the hypothesis of the SBV circulation in Southern Spain during the same period when first outbreaks were detected in Northern Europe [3,4]. In this context, retrospective studies conducted in sheep [28] and wild ruminant species [5] in the same

region, indicate that SBV had been silently circulating in Spain since summer 2011, when the virus was first identified in Germany [3].

The high individual and herd seroprevalence found in domestic ruminants in the present study (54.4% and 89.7%, respectively), indicate that SBV was widespread across the affected region one year after the outbreak. Our results are in keeping with those observed after the first epidemic outbreaks reported in other European countries such as The Netherlands [29], Belgium [30], France [31] and Poland [32]. In Spain, other SBD outbreaks as well as presence of anti-SBV antibodies were reported in domestic ruminants in Central and North-eastern regions in early 2013 [15,16]. During the last few years, SBV exposure has been also confirmed in wild ruminant species in different regions of this country included the study area [33,5,34], which suggests an endemic circulation of the virus in these species [5]. Further research is required to establish the spatio-temporal distribution of SBV in domestic ruminants in Spain.

The GEE model identified the species, age and absence of animal insecticide treatment as risk factors potentially associated with SBV exposure in domestic ruminants in the studied area (Table 2). The seroprevalence was significantly higher in cattle compared with small ruminant species, which is in agreement with previous reports [15,35]. Although biting midges can feed on a wide range of vertebrate hosts, cattle have been identified as the preferred species for *Culicoides* spp. feeding [36]. Among other possible factors, the presence of thick wool in sheep makes it difficult for midges to reach the skin, which could also explain the lower seroprevalence detected in this species [37]. Likewise, although little is known about *Culicoides* spp. preference for goats, their species-specific odour has been proposed to explain the lower prevalence of antibodies against SBV observed in goats compared with other ruminant species [37].

A significantly higher seropositivity to SBV was found in adult animals, which may be associated with the greater exposure of this age class over time and the lifelong persistence of SBV antibodies. In this context, anti-SBV antibodies have been detected in naturally infected ruminants up to six years post-infection [38]. The increase in seroprevalence with age has been previously observed in domestic [39] and wild ruminants [5]. The treatment of animals with insecticides was shown to be a relevant protective factor against SBV infection. Even though a wide-scale control of vectors through treatment on the environment is considered unfeasible, the use of pyrethroids on animal hosts has been highlighted as a useful control measure for *Culicoides* spp. biting prevention [40,41].

5. Conclusions

In conclusion, both pathological and molecular findings confirm the first clinical SBV outbreak in Spain in March 2012, few months after the virus was first identified in Germany. The high individual and herd seroprevalences detected evidence a widespread circulation of SBV in domestic ruminants in the same region where the first outbreak was reported. Further studies are needed to determine the spatio-temporal trend of SBV in domestic ruminants in Spain.

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Figure captions

Figure 1. Stillborn lamb infected with Schmallenberg virus (SBV) presenting skeletal muscular atrophy, brachygnathia inferior, generalised arthrogryposis of the appendicular skeleton (arthrogryposis multiplex congenita) and vertebral column malformation, including torticollis and kyphoscoliosis (A). Severe cerebellar hypoplasia and congestion of the meninges in SBV-infected ovine fetus (B). Transverse sections of skeletal muscle showing a severe myofibrillar hypoplasia (C), where most muscle fibres were missing and replaced in part (arrowheads) or totally by adipose tissue (arrows) (inset). Haematoxylin and eosin staining (HES), bar = 300 µm (C). Heart, myocardial oedema and cardiomyocytes with a great amount of interspersed blood capillaries (D). HES, bar = 50 µm.

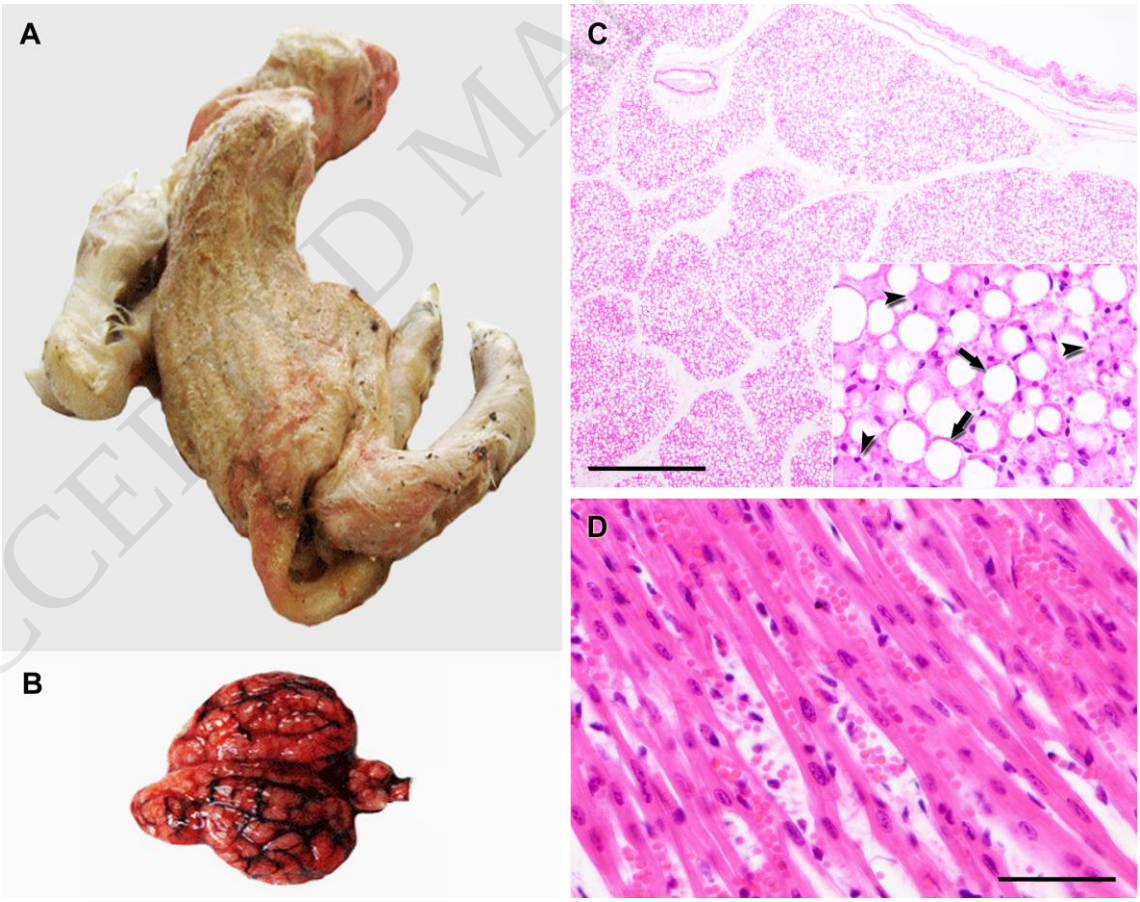

Figure 2. Map of the study area (Cordoba province, Southern Spain) showing the first Schmallenberg virus (SBV) outbreak in Spain (blue star) and the analysed herds (dots). Colour gradient in dots indicates the within herd SBV seroprevalence.

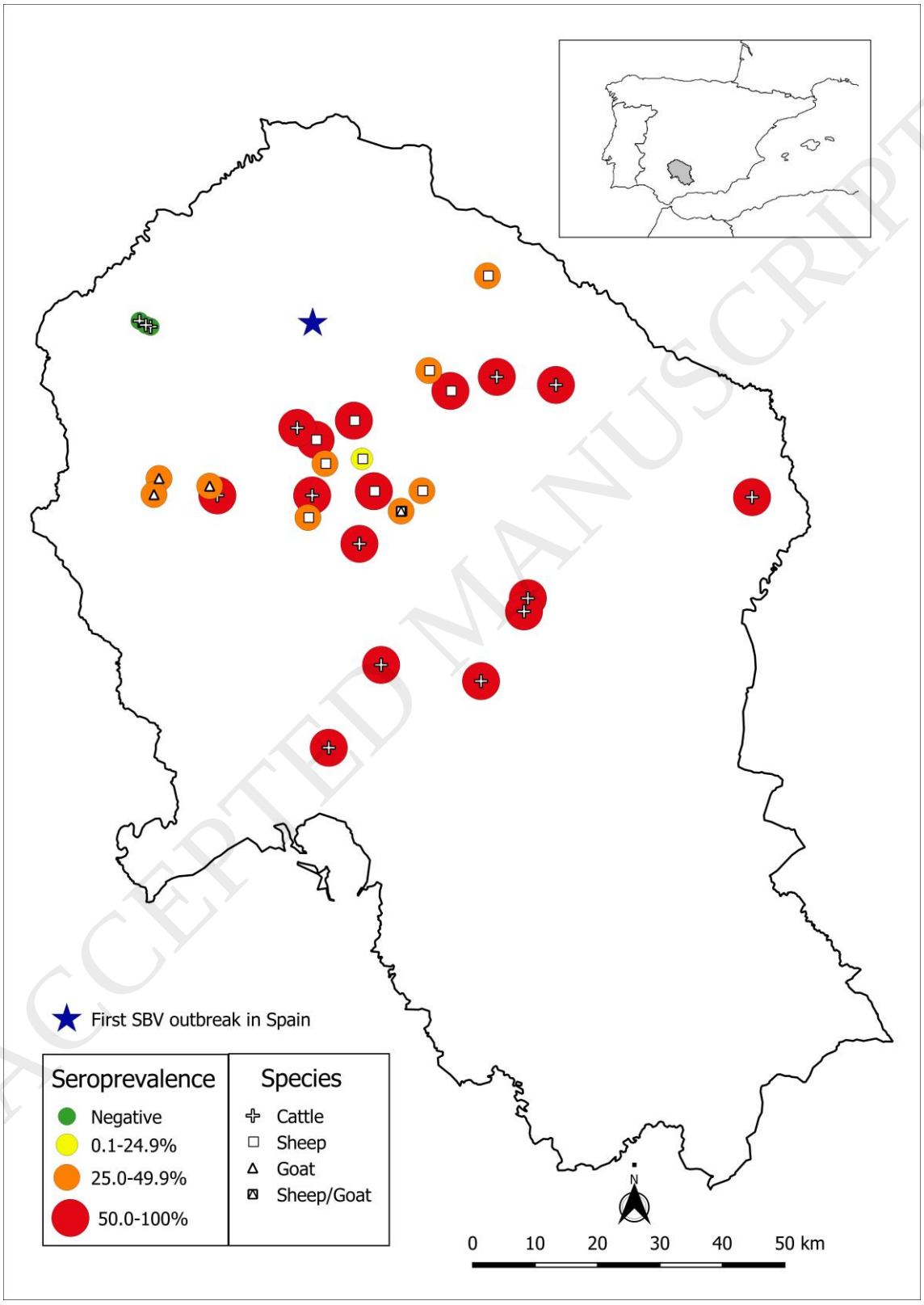

Table legends

Table 1. Seroprevalence of Schmallenberg virus in domestic ruminants from Cordoba province (Southern Spain).

Variable	Exposure levels	% bELISA positive	Number/overall	*p*-value
Species	Cattle	70.6	149/211	<0.001
	Sheep	46.0	93/202	
	Goat	34.8	31/89	
Sex	Male	34.2	13/38	0.009
	Female	56.0	260/464	
Age	Non-adults	13.2	7/53	<0.001
	Adults	59.2	266/449	
Surface area for grazing (hectares)	<200	59.0	167/283	0.018
	>200	48.4	106/219	
Feeding on ground	Yes	66.0	105/159	<0.001
	No	49.0	168/343	
Feeding at stubble field	Yes	42.1	93/221	<0.001
	No	64.1	180/281	
Feeding on feeders	Yes	50.5	98/194	0.167
	No	56.8	175/308	
Presence of natural water points	Yes	52.2	206/395	0.054
	No	62.6	67/107	
Presence of artificial water points	Yes	57.2	258/451	<0.001
	No	29.4	15/51	
Agriculture uses of land	Yes	51.1	48/94	0.474
	No	55.1	225/408	
Temporal sheltering	Yes	51.1	92/180	0.271
	No	56.2	181/322	
Cleaning protocol	Yes	35.6	16/45	0.008
	No	56.2	257/457	
Animal insecticide treatment	Yes	34.4	21/61	0.001
	No	57.1	252/441	
Deworming program	Yes	54.3	266/490	0.781
	No	58.3	7/12	
Rodent control program	Yes	53.3	72/135	0.775
	No	54.8	201/367	

Disinfection protocol	Yes	70.0	21/30	0.077
	No	53.4	252/472	
External replacement	Yes	37.7	26/69	0.003
	No	57.0	247/433	
Presence of other domestic ruminants	Yes	53.9	207/384	0.699
	No	55.9	66/118	
Presence of wild ruminants	Yes	51.0	227/445	<0.001
	No	80.7	46/57	
Distance to the nearest farm (meters)	>500	54.5	73/134	0.947
	500-1000	53.8	147/273	
	<1000	55.8	53/95	

Table 2. Generalized estimating equation analysis of potential risk factors associated with Schmallenberg virus exposure in domestic ruminants from Cordoba province (Southern Spain).

Variable	**Categories**	***β* (S.E.)**	**Sig.**	**df**	**OR CI$_{95\%}$**
Species	Goat	[a]	[a]		[a]
	Sheep	0.31 (0.33)	0.343	1	1.37 (0.72-2.61)
	Cattle	2.28 (0.56)	<0.001	1	9.74 (3.22-20.51)
Age	Non-adults	[a]	[a]		[a]
	Adults	2.38 (0.65)	<0.001	1	10.6 (2.98-37.5)
Animal insecticide treatment	No	[a]	[a]		[a]
	Yes	-2.01 (0.51)	<0.001	1	0.13 (0.05-0.37)

[a] Reference category.